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Assessment of Neurobehavioral Toxicity of *Dendrophthoe falcata* (L.f) Ettingsh in Rats by Functional Observational Battery after a Subacute Exposure

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ABSTRACT

The hemi-parasitic plant *Dendrophthoe falcata* (L.f) Ettingsh (Loranthaceae) of the order Santalales, is used ethnomedicinally for treating ulcers, asthma, impotence, paralysis, skin diseases, and wounds. The aerial parts are also used in menstrual troubles, psychic disorders, pulmonary tuberculosis, consumption and mania by the tribal of India. In this context, the plant requires the validation of any potential toxicity before therapeutic promotion. The aim of the present study was to evaluate the neurobehavioral toxicity of the hydroalcoholic extract from *D. falcata* growing on the host plant *Azadirachta indica*, after subacute exposure. The LD₅₀ was assessed in female wistar rats and was found to be 4550mg/kg by oral route. The plant extract was administered in three different doses i.e. 250mg/kg, 475mg/kg and 950mg/kg body weight/day for a period of four weeks. At the end of the exposure, behavioural and functional parameters were assessed in a functional observational battery (FOB) and motor activity was measured in an open field. A decrease in the arousal level was observed in experimental groups. Also, the total number of urine spots increased in a dose dependent manner for extract treated groups. Our results suggest that hydroalcoholic extracts from aerial parts of *D. falcata* should be relatively free from any serious neurobehavioral toxicity and safe to use.

KEY WORDS: *Dendrophthoe falcata* (L.f) Ettingsh, Hydroalcoholic extracts, Sub-acute exposure, Neurobehavioral toxicity, FOB.

INTRODUCTION

Dendrophthoe falcata (L.f) Ettingsh (Loranthaceae), commonly known as 'Banda' (Hindi) is a evergreen shrub with bark smooth grey, leaves opposite unequal, thick 1.6 - 25.4 cm long, flowers single, orange-red or scarlet softly pubescent, berries soft ovoid-oblong, 1.3cm diameter and indigenous to India, Srilanka, Thailand, Indo-china, Australia (1). It is a large bushy parasitic plant that grows on a variety of host plants in deciduous forests and the entire plant is medicinally important (2). The aerial parts are used in wounds, menstrual troubles, asthma, psychic disorders, pulmonary tuberculosis, consumption and mania by the tribal of India (3, 4, 5, 6). Leaf paste is used in skin diseases (7). Its paste is applied on boils, setting dislocated bones and extracting pus (8). The plant has been scientifically proved to have anti-

lithiatic, diuretic, cytotoxic and immunomodulatory activities (9, 10). In previous phytochemical studies, *D. falcata* have been reported to contain several cardiac glycosides, flavonoids, and pentacyclic triterpenes (11, 12).

In order to continue assessing the potential therapeutic use, it is necessary to investigate their safety through toxicity studies. In the present work, we evaluated the sub-acute neurobehavioral toxicity of hydroalcoholic extract from aerial parts of *D. falcata* growing on the host plant *Azadirachta indica*, in rats by means of a functional observational battery (FOB) (13) and by assessing the motor activity in an open field (14). Functional observational battery evaluations in animals are similar to clinical neurologic examinations in humans in that they rate the presence

and severity of behavioural and neurologic dysfunction. FOB evaluations in screening typically assess several neurobiologic domains including neuromuscular (weakness, incoordination, abnormal movements, gait, motor seizures, myoclonia, rigidity and tremors), sensory (auditory, visual, and somatosensory), and autonomic (pupil response and salivation) functions.

MATERIALS AND METHOD

Plant material

Fresh aerial parts of *D. falcata* were collected in December 2007 from the thick forest areas of Similipal biosphere reserve, Mayurbhanj district of Orissa, India. *Dendrophthoe falcata*(L.f)Ettingsh (Loranthaceae) was authenticated by Dr. N.K. Dhal, Department of Natural products, Regional Research Laboratory (RRL), Bhubaneswar, India. Two sets of herbarium voucher specimens were mounted and one set was deposited at RRL, Bhubaneswar vide access no 9996 and one set has been preserved in our laboratory for future reference.

Preparation of extracts

The aerial parts were air-dried, pulverized to a coarse powder in a mechanical grinder, passed through a 40 mesh sieve and extracted in a soxhlet extractor with ethanol-water (8:2). The extract was decanted, filtered with Whatman No. 1 filter paper and concentrated at reduced pressure below 40 °C through rota vapor to obtain dry extract (20.6% w/w). *Dendrophthoe falcata* hydroalcoholic extract (DFHE) was kept at 4°C.

Animals

Female Wistar rats of 8 weeks old were used for subacute exposure. They were maintained under constant temperature conditions (22 ± 1°C) in a 12-h light:12-h dark cycle (Light on at 07:00), provided with standard food and water ad lib. The experiments were conducted in accordance with the institute's ethical committee approval and guidelines Reg no. 621/02/ac/CPCSEA of Birla Institute of Technology, Mesra, India under the proposal approval no. BIT/PH/IAEC/05/2008.

LD₅₀ determination

From the acute toxicity study data it was found that at the dose level of 3500mg/kg there was no mortality and at 6000mg/kg all the animals were dead. LD₅₀ determination of DFHE was performed as described by Graphical method (15). Different doses of 3.5, 4, 4.5, 5, 5.5 and 6g/kg were administered orally to the animals of six groups, each containing four animals. The toxicological effect was assessed on the basis of mortality after 24h, which was expressed as an LD₅₀ value. The percentage of mortality was converted to

Probits and the values were plotted against log dose. The LD₅₀ was the dose intersected by Probit 5.

Subacute exposure

The plant extract was suspended in 0.3% w/v Sodium carboxy methyl cellulose (Na CMC) in distilled water for experimental use. Three groups of six animals received a daily dose of 250mg/kg of body weight (b.w.) (~ 0.05 × LD₅₀), 475mg/kg b.w. (~ 0.1 × LD₅₀), and 950mg/kg b.w. (~ 0.2 × LD₅₀) of DFHE during a 28-day period. Another group formed by 6 rats was used as a control to which vehicle (Na CMC) was administered. In each case the product volume administered was 10ml/kg body weight. The parameter measured during the exposure period was body weight in each alternate day. At the end of the exposure, behavioral and functional parameters and motor activity were assessed in all animals.

Functional observational battery

The FOB includes a thorough description of the animals' appearance, behaviour and functional integrity (US EPA, 1998). This was assessed through observations in the home cage, while animals were moving freely in an open field, and through manipulative tests. Procedural details and scoring criteria for the FOB protocol have been according to McDaniel and Moser, 1993 (16) with some modifications for rats (Appendix 1).

Briefly, measurements were first carried out in the home cage. The observer recorded each animal's posture, activity and palpebral closure. The presence or absence of tremors and convulsions were noted and, if present, described. The presence or absence of spontaneous vocalizations and biting was also noted. The observer then removed the animal, rating the ease of removal and handling. The presence or absence of hind limb flexor resistance and pressure grade was also noted. Palpebral closure and any lacrimation or salivation were rated. Other abnormal clinical signs were also recorded. The animal was next placed in an open field arena having a piece of clean absorbent paper on the surface and allowed to freely explore for 2 min. During that time, the observer ranked the rat's arousal, gait score, activity level and rears as well as any abnormal postures, unusual movements and stereotypy. At the end of the 3 min, the number of fecal boluses and urine pools and presence or absence of diarrhoea on the absorbent paper was recorded. Next, sensorial responses were ranked according to a variety of stimuli (click stimulus using a metal clicker, approach and touch rump with a blunt object, pinch of the tail using metal tweezers, constriction of the pupil to a penlight stimulus and touch of the corner of the

eye with a fine cotton thread). Also, several motor reflexes were evaluated (forelimb hopping, proprioceptive positioning, forelimb and hindlimb extensions). Degree of surface and aerial righting were rated next. In landing foot splay, the tarsal joint pad of each hindfoot was marked with ink and the animal was then dropped from a height of 30 cm onto a recording sheet. This procedure was repeated three times. The distance from center-to-center of the ink marks was measured (cm) and the average of the three splay values was used for statistical analysis.

Motor activity

An open field of 77 cm × 55 cm × 7 cm [l × w × h] whose floor was divided into 12 cm × 12 cm squares by black lines was used. The number of squares entered with all four paws, rearing, grooming and fecal boluses were scored each 5 min for 15 min. After each animal was removed, the open field was carefully cleaned with a damp cloth.

Statistical analysis

Behavioural test measures in FOB were continuous (providing interval data or count data), ranked (ranked based on a defined scale), descriptive or quantal (presence or absence of sign). Count, ranked and interval data were submitted to a one-way ANOVA. Where as, the difference between groups in each case were analyzed by Dunnet's *t*-rest. In all cases, resulting probability values < 0.05 were considered significant.

RESULTS

LD₅₀ determination

The LD₅₀ was also determined by the graphical method and was found to be 4550mg/kg (Table 1, Fig.1).

Functional observational battery

In the subacute exposure of hydroalcoholic extract from the aerial parts of *Dendrophthoe falcata* revealed no significant differences in body weight of all the animals (data not shown). The data obtained in the FOB are shown in Table 1 & 2. DFHS exposure to the rats produced no alterations in the parameters evaluated in the home cage or during the manipulative tests. Also, no abnormal clinical signs were observed in control and experimental groups. However, in the open field arena both experimental groups exhibited a significant decrease in the arousal level ($p < 0.05$) compared to control groups. The other parameters evaluated in the open field arena were not altered in the animals exposed. Motor activity evaluations in the square open field indicated that the subacute exposure did not modify the number of squares crossed during a total of 15 min on day 28 (Fig.2). The ANOVA for repeated measures for comparisons in the number of squares crossed in each 5 min period, did not show significant differences in the number of squares. Moreover, the total number of urine spots were significantly increased in DFHE treated group. The effect followed the expected exposure-response relationships with less significant ($p < 0.05$) effect in the low exposure (250mg/kg) group, more significant ($p < 0.01$) effect in the high exposure (950mg/kg) group and transitional response with statistically significant findings, in the intermediate (475 mg/kg) group. When we analysed the emotional parameters as the number of grooming and fecal boluses, no measures demonstrated any significant differences between control and all the experimental groups.

Table 1: Determination of LD₅₀ values for the hydroalcoholic extract of *Dendrophthoe falcata*.

Dose (mg/kg body wt.)	Log dose	Percent mortality (after 24 h)	Corrected mortality (%)	Probit
3500	3.544	0	4.1	3.25
4000	3.602	17	17	4.05
4500	3.653	33	33	4.56
5000	3.699	50	50	5.00
5500	3.740	83	83	5.95
6000	3.778	100	95.83	6.75

Table 2: Summary of the observed parameters in Functional Observational Battery after *Dendrophthoe falcata* dosing.

Endpoints	Sub-acute exposure			
	Control	250 mg/kg b. w. (DFHS)	475 mg/kg b. w. (DFHS)	950 mg/kg b. w. (DFHS)
Home cage Assessment				
Normal body posture (D) (%)	100	100	100	100
Palpebral closure (R)	1.0	1.0	1.0	1.0
Convulsions or tremors (D) (%)	0.0	0.0	0.0	0.0

Biting (D) (%)	0.0	0.0	0.0	0.0
Vocalization (Q) (%)	0.0	0.0	0.0	0.0
Hand held observations				
Easy of removal from cage (R)	1.3±0.21	1.3±0.21 ^{ns}	1.17±0.16 ^{ns}	1.0±0.0 ^{ns}
Ease of handling rat in hand (R)	1.17	1.0	1.0	1.0
Lacrimation (R)	1.0	1.0	1.0	1.0
Salivation (R)	1.0	1.0	1.0	1.0
Fur appearance (D) (%)	100	100	100	100
Open Field Observations				
Latency (to leave the middle square) (secs)	2.96±0.36	2.5±0.43 ^{ns}	2.67±0.33 ^{ns}	4.0±0.26 ^{ns}
Total No. of grooming episodes (C)	4.0±0.58	3.0±0.5 ^{ns}	3.0±0.51 ^{ns}	3.3±0.61 ^{ns}
Arousal (R)	4.17±0.16	3.17±0.4 ^{ns}	2.5±0.56*	1.83±0.31**
Gait description (D)	1.0	1.0	1.0	1.0
Total no. of fecal bolus (C)	1.5±0.34 ^{ns}	1.17±0.16 ^{ns}	1.5±0.22 ^{ns}	1.3±0.21 ^{ns}
Diarrhoea (D)	0.0	0.0	0.0	0.0

DFHS – *Dendrophthoe falcata* hydroalcoholic extract; Descriptive (D) and quantal (Q) data expressed as percentage of incidence; ranked (R) data expressed as mean ± standard error mean (SEM) of the scale used (Appendix -1); continuous or count data (C) and interval data (I) expressed as mean ± SEM; R, C & I data were subjected to one-way ANOVA followed by Dunnett's *t*-test for comparison with the control group; ns: non significant difference, **: $p < 0.01$, *: $p < 0.05$.

Table 3: Summary of the observed parameters in Functional Observational Battery after *Dendrophthoe falcata* dosing.

Endpoints	Sub-acute exposure			
	Control	250mg/kg b. w. (DFHS)	475mg/kg b. w. (DFHS)	950mg/kg b. w. (DFHS)
Total no. of urine spots (C)	1.67±0.17	2.33±0.3*	2.5±0.22*	2.83±0.4**
Reflexes				
Click response (R)	1.66 ±0.2	1.5±0.22 ^{ns}	1.3±0.21 ^{ns}	1.17±0.16 ^{ns}
Approach response (R)	1.5±0.22	1.67±0.21 ^{ns}	1.3±0.2 ^{ns}	1.16±0.17 ^{ns}
Touch response (R)	1.3±0.2	1.16±0.16 ^{ns}	1.3±0.21 ^{ns}	1.0±0.0 ^{ns}
Tail Pinch (R)	2.16±0.17	2.0±0.0 ^{ns}	2.0±0.0 ^{ns}	1.83±0.16 ^{ns}
Pupil response (Q) (%)	100	100	100	100
Eye blink response (Q) (%)	100	100	100	100
Gait Analysis				
Fore limb extension (Q) (%)	100	100	100	100
Hind limb extension (Q) (%)	100	100	100	100
Gait scoring (C)				
Length	9.83±0.106	9.317±0.302 ^{ns}	9.18±0.217 ^{ns}	9.57±0.123 ^{ns}
Width	3.367±0.07	3.08±0.087 ^{ns}	3.16±0.14 ^{ns}	3.27±0.115 ^{ns}
Angle	106.3±2.29	105±1.9 ^{ns}	109.1±1.01 ^{ns}	105.6±1.6 ^{ns}
Landing foot splay (I)	9.93±0.138	9.73±0.158 ^{ns}	9.55±0.209 ^{ns}	9.9±0.093 ^{ns}
Righting reflex (R)	1.0	1.0	1.0	1.0
Rectal body temperature (10sec)	36.2±0.4	34.38±0.43 ^{ns}	36.03±0.25 ^{ns}	35.1±0.41 ^{ns}

DFHS – *Dendrophthoe falcata* hydroalcoholic extract; Descriptive (D) and quantal (Q) data expressed as percentage of incidence; ranked (R) data expressed as mean ± standard error mean (SEM) of the scale used (Appendix -1); continuous or count data (C) and interval data (I) expressed as mean ± SEM; R, C & I data were subjected to one-way ANOVA followed by Dunnett's *t*-test for comparison with the control group; ns: non significant difference, **: $p < 0.01$, *: $p < 0.05$.

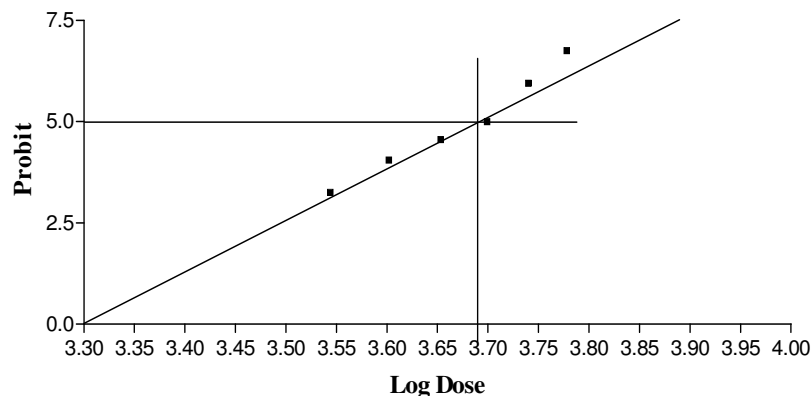


Figure 1: Determination of LD_{50} value for the hydroalcoholic extract of *Dendrophthoe falcata* administered to rats for 24h, using a graphical method. $LD_{50} = \text{Log } 3.657 = 4539 \text{ mg/kg} \sim 4550 \text{ mg/kg}$.

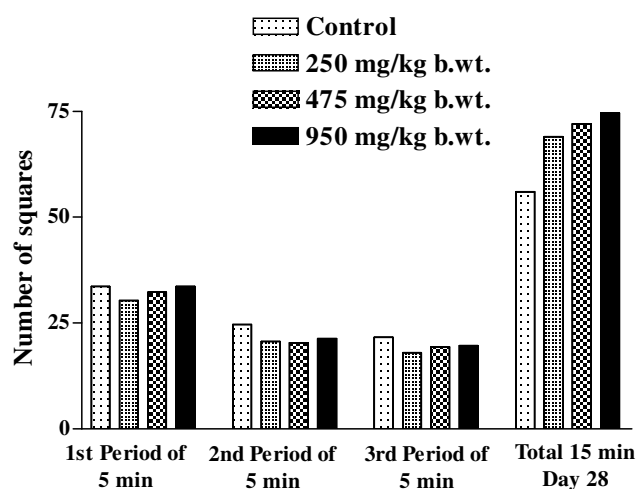


Figure 2: Motor activity evaluated in the open field after the subacute exposure. Data were calculated as $\text{mean} \pm \text{S.E.M.}$ of the number of squares entered by the rat, recorded during each period of 5 min (data analysed using ANOVA for repeated measures followed by Dunnett's *t*-test and in the complete period of 15 min at the end of the treatment (day 28)).

DISCUSSION

The overall objective of this study was to evaluate the behavioral effects of DFHE exposure in rats. We focused our attention on CNS since the plant is used for the treatment of some psychic disorders. It is also widely used for the treatment of other diseases, so the neurobehavioral parameters were observed to see whether the plant is having any inherent toxicity which if present would make it unsuitable for any therapeutic promotion.

Decrease in the arousal level observed in experimental groups in subacute exposure indicates that the plant may have some depressant like activity, which makes it suitable for the treatment of mania (4, 5). Among the behavioral measurement the most consistent finding was more urine spots in the open field arena, which might be indicating its diuretic property (9), but there were no effects on general measures of

responding, stimulus control, or disinhibition. The summary of the rodent data publicized that the hydroalcoholic extract of *D. falcata* might have no effect on neural integrity (13) which was checked through FOB test and motor activity test indicate that it might be free from neurobehavioral dysfunction (14).

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Appendix 1

FUNCTIONAL OBSERVATIONAL BATTERY

Study No. _____ Rat No. _____ Sex _____ Hour _____
Date _____ Performed By _____ Recorded By _____

Home Cage Assessment

Posture: (D) (Choose one)

1. asleep, lying on side, or curled up
2. lying on side, resting but awake
3. sitting or standing normally
4. rearing
5. hunched over

Palpebral Closure: (R) (Choose one)

1. eyelids wide open
2. eyelids slightly drooping
3. ptosis: drooping eyelids (half closed)
4. eyelids completely shut

Convulsions or tremors: (D) (choose one)

1. absent
2. present
 - a) clonic (contractions followed by relaxation)
 - b) tonic (constant contraction and extension of hind limb muscles)

Biting: (D) (Choose one)

1. none
2. biting of cage
3. self-destructive

Vocalizations: (Q) (choose one)

1. absent
2. present

Hand Held Observation

Ease of removal from cage: (R) (choose one)

1. easy: little or no vocalization, without resistance or only slight resistance to being picked up
2. moderately difficult; rat rears, often following investigator's hand
3. difficult; runs around cage, is hard to grab, with or without vocalization

Ease of handling rat in hand: (R) (choose one)

1. easy, alert, limbs may be pulled against body
2. moderately easy; vocalization, without resistance to being handled
3. difficult; squirming, twisting, attempting to bite, with or without vocalization

Lacrimation: (R) (choose one)

1. none
2. slight
3. severe

Salivation: (R) (choose one)

1. none
2. slight
3. severe

Fur appearance: (D) (choose one)

1. normal
2. slightly soiled
3. very soiled, crusty
4. rough

Open Field Observations (2 minutes)

1. Latency (to leave the middle square) (secs) _____
2. Total No. of squares entered (hand tally counter) _____
3. Total No. extra lines crossed _____
4. Sniffing movement (time) _____
5. Freezing (time) _____
6. Total No. of rears (C) _____
 - 1) supported by cage sides _____
 - 2) unsupported _____
7. Total No. of grooming episodes (C) _____

Arousal: (R) (choose one)

1. very low (little or absent)
2. low (some head or body movement)
3. somewhat low (some exploratory movements with periods of immobility)
4. normal (alert, exploratory movements)
5. somewhat high (slight excitement, sudden darting, or freezing)
6. very high (hyper-alert, excited, sudden bouts of running or body movements)

Gait description: (D) (Choose one)

1. normal
2. impairment
 - a) uncoordinated movement (i.e., ataxia)
 - b) walking on toes
 - c) splayed hind limbs
 - d) exaggerated hind limb flexion
 - e) staggered gait
 - f) dragging hind limbs
 - g) unable to walk
 - h) other (e.g. rolling over, sleeping, convulsions)

Total No. of fecal boluses (C) _____

Diarrhoea: (D) (choose one)

1. present
2. absent

Total No. of urine spots (C) _____

Reflexes

1. click response: (R) (inside cage) (choose one)
 - 1) no reaction
 - 2) slightly reaction, ear flick or some evidence that snap was heard
 - 3) more energetic response than (2), may include vocalization
 - 4) freezes, actual muscle contractions
 - 5) bizarre reaction: jumps, bites, attacks
2. Approach response: (R) (approach rat head on with a blunt object held approximately 3 cm from its face for a 4-second period) (choose one)
 - 1) no reaction
 - 2) slow approach, sniffing, or turning away
 - 3) more energetic response than (2), may include vocalization
 - 4) freezes, actual muscle contractions
 - 5) bizarre reaction: jumps, bites, or attacks
3. Touch response: (R) (touch rump with a blunt object) (choose one)
 - 1) no reaction
 - 2) rat may slowly turn, walk away
 - 3) more energetic response than (2), may include vocalization
 - 4) freezes, actual muscle contractions
 - 5) bizarre reaction: jumps, bites, or attacks
4. Tail pinch: (R) (metal tweezers are used to squeeze the tail approximately 5cm distal to the body) (choose one)
 - 1) no reaction
 - 2) rat may turn walk away
 - 3) more energetic response than (2), may include vocalization
 - 4) freezes, actual muscle contractions
 - 5) bizarre reaction: jumps, bites, or attacks
5. Pupil response: (Q) (beam from pen light is brought in from the side of the rat's head and changes in direct and consensual pupil size are noted) (choose one)
 - 1) pupil response present
 - 2) no pupil response
6. Eye blink response: (Q) (corner of eye is touched gently with a cotton thread) (choose one)
 - 1) pupil response present
 - 2) no pupil response
7. Gait analysis:
 - a) Fore limb extension: (Q) (animal is held by the tail at height of 60 cm and lowered toward table top; presence of normal fore limb extension is noted) (choose one)
 - 1) Fore limb extension present
 - 2) No fore limb extension
 - b) Hind limb extension: (animal is placed on table top and lifted; presence of normal hind limb extension is noted) (Choose one)
 - 1) hind limb extension present
 - 2) no hind limb extension
 - c) Gait scoring: (C) (1 trial) (after staining rat's hind feet with ink, animal is allowed to walk through enclosed corridor with paper-covered floor. When 2 consecutive strides are obtained, the stride length, width, angle between consecutive steps on contralateral sides are calculated)
Length (cm) _____ Width (cm) _____
Angle _____
 - d) landing foot splay: (I) (2 trials) (after staining rat's hind feet with ink, animal is held horizontally 30 cm above a table covered with paper. The rat is dropped and the distance between the fourth digits of each hind foot is recorded.)
Trial 1 (cm) _____ Trial 2 (cm) _____
 - e) Righting reflex: (R) (hold rat in supine; drop approximately 30 cm and score ease of landing) (choose one)
 1. normal
 2. slightly uncoordinated
 3. rolls on side
 4. rolls on back

Physiologic Measurements

Body weight (grams) (I): _____

Rectal body temperature (10 sec): _____

Comments:

Abbreviations:

D = descriptive

R = rank order

Q = quantal

C = count data

I = interval data